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14. ABSTRACT Docetaxel (DTX), a semi-synthetic analog of paclitaxel, has emerged as the standard of care for chemotherapy of hormone-resistant prostate cancer. Docetaxel confers its anti-neoplastic activity by inhibiting microtubule depolymerization, which leads to G2/M mitotic arrest and subsequent apoptosis (1). However, most patients treated with DTX ultimately develop resistance to the drug and succumb to the disease (2). Therefore, understanding the mechanisms underlying DTX resistance is a priority area in prostate cancer research. Previously, it was reported that CXCL12/CXCR4 signaling play an important role in microtubule organization in immune cells (3) and its inhibition induced mitotic catastrophe (G2/M arrest) in ovarian cancer cells (4). Based on these earlier observations, we hypothesized that CXCL12-CXCR4 signaling axis would promote docetaxel resistance by counteracting the microtubule stabilizing action of docetaxel.					
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Exploring a novel mechanism of docetaxel resistance in prostate cancer- Progress Report

INTRODUCTION: Docetaxel (DTX), a semi-synthetic analog of paclitaxel, has emerged as the standard of care for chemotherapy of hormone-resistant prostate cancer. Docetaxel confers its anti-neoplastic activity by inhibiting microtubule depolymerization, which leads to G2/M mitotic arrest and subsequent apoptosis (1). However, most patients treated with DTX ultimately develop resistance to the drug and succumb to the disease (2). Therefore, understanding the mechanisms underlying DTX resistance is a priority area in prostate cancer research. Previously, it was reported that CXCL12/CXCR4 signaling play an important role in microtubule organization in immune cells (3) and its inhibition induced mitotic catastrophe (G2/M arrest) in ovarian cancer cells (4). Based on these earlier observations, we **hypothesized** that CXCL12-CXCR4 signaling axis would promote docetaxel resistance by counteracting the microtubule stabilizing action of docetaxel. This hypothesis is being tested in two specific aims:

- 1) To investigate the role of CXCL12/CXCR4 signaling in microtubule dynamics of prostate cancer cells.
- 2) To examine if activation of this signaling node restricts docetaxel-induced microtubule stability and toxicity in prostate cancer cells *in vitro*.

RESEARCH PROGRESS:

Task 1: To examine the effect of CXCL12/CXCR4 signaling on microtubule dynamics in prostate cancer cells.

First, we examined the basal expression level of CXCR4 and CXCL12, a sole ligand of CXCR4; in four

prostate cancer (LNCaP, C4-2, PC3 and DU145) and two normal prostate epithelial (RWPE1 and RWPE2)

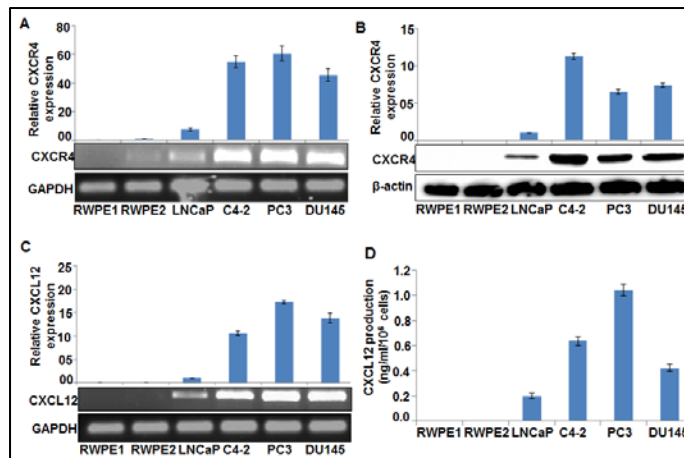


Figure 1: CXCL12 and CXCR4 expression in prostate cancer cells. Expression of CXCR4 (A & B) and CXCL12 (C & D) was examined by RT-PCR and immunoblot assays. Data show an aberrant expression of CXCR4 and very low levels of CXCL12 in all prostate cancer cells whereas, no expression of CXCR4 as well as CXCL12 was observed in normal prostate epithelial cells (n=3)

cell lines cells at transcription and protein levels by quantitative RT-PCR and immunoblot /ELISA analyses, respectively. Our data demonstrate an expression of CXCR4 (**Figure 1 A and B**) and very low level of CXCL12 (0.2 -1.1 ng per ml per 10^6 cells) (**Figure 1 C and D**) in all the tested prostate cancer cell lines (LNCaP, C4-2, DU145 and PC3). However, no or negligible expression of both CXCR4 and CXCL12 was noted in prostate epithelial cell lines (RWPE1 and

RWPE2) (**Figure 1**). Taken together, our data show that CXCR4 is aberrantly overexpressed in prostate cancer cells, while they produce very low level of CXCL12.

To investigate the effect of CXCL12 treatment on the microtubule dynamics in prostate cancer (C4-2 and PC3) cells, we treated cells with various doses of CXCL12 (0-200 ng per mL) for 24h and examined visual changes in morphology under phase-contrast light microscope. No significant changes were observed in the morphology under light microscope of both the prostate cancer (C4-2 and PC3) cells after CXCL12 treatment as compared to untreated cells. Similarly, no or weak signal was detected when we examined acetylation and dephosphorylation of alpha-tubulin in the CXCL12-treated or untreated cells by immunoblot analysis using specific antibodies. These data are suggestive of the fact that microtubules undergo dynamic assembly/disassembly under normal growth conditions in prostate cancer cells.

Task 2: To investigate the effect of CXCR4 activation on docetaxel-induced microtubule sensitivity and growth suppression.

To investigate the chemoprotective effect of CXCL12 in prostate cancer cells, C4-2 and PC3 cells were grown in 96 well plate and treated with different doses of DTX (0-30 nM) in presence or absence of CXCL12 (100 ng/ml). After 48 h of treatment, cell viability was evaluated by WST1 assay. Our data show that CXCL12 treatment suppresses DTX-induced cytotoxicity in PCa cells. Percent viability of C4-2 and PC3 cells treated with 15 nM of DTX was > 90 % in presence of CXCL12, while it was ≈ 45 and 64 % in the absence of CXCL12 (**Figure 2**). To further

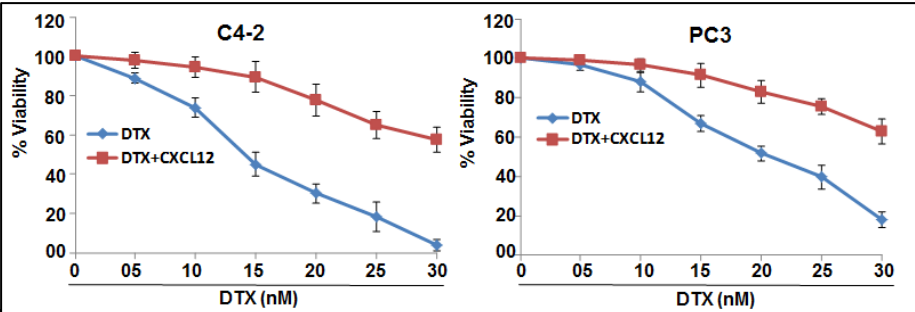


Figure 2: CXCL12 rescue the docetaxel-induced cytotoxicity: C4-2 (**A**) and PC3 (**B**) prostate cancer cells were treated with various doses of docetaxel (DTX) (0–30 nM) in the presence and absence of CXCL12 (100 ng/mL). After 48 h, viability of cells was examined WST-1 assay. Data are presented as percent viability with respect to untreated or CXCL12 only-treated cells. Data show that CXCL12 treatment protects PCa cells from DTX-induced cytotoxicity.

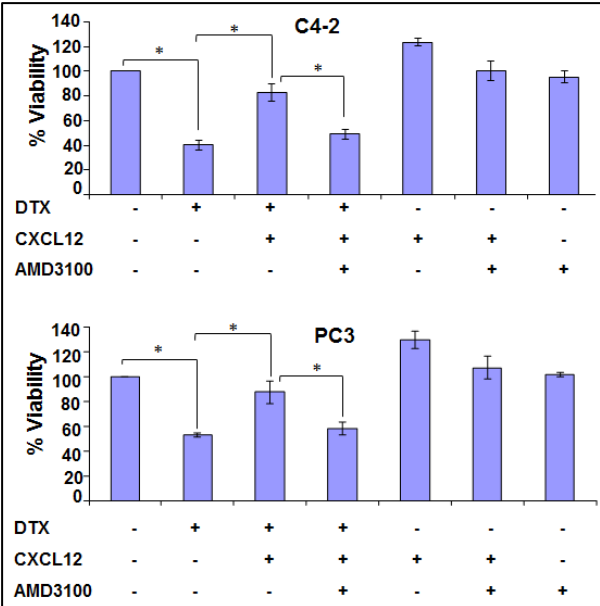


Figure 3: Effect of CXCR4 antagonist on the chemoprotective effect of CXCL12. C4-2 (**Upper panel**) and PC3 (**lower panel**) cells were treated with the AMD3100, a CXCR4 antagonist, for 1h prior to the treatment of CXCL12 followed by DTX treatment. After 48 h of treatment, viability of cells was examined by WST-1 assay. Bars represent the average of triplicates ± S.D.; * p<0.01). Data show that chemoprotective effect of CXCL12 was reversed by the pre-treatment of AMD3100.

confirm the role of CXCL12/CXCR4 signaling in DTX-resistance, we treated PCa cells with AMD3100, a small molecule antagonist of CXCR4, prior to the treatment of CXCL12 and DTX. Resulting cell viability data show that CXCL12-induced chemopreventive effects in C4-2 and PC3 cells were abolished by the AMD3100 pre-treatment (**Figure 3**). Altogether, these findings suggest that CXCL12 treatment decreases DTX-induced cytotoxic effect in pancreatic cancer cells.

Induction of microtubule polymerization followed by G2/M cell cycle arrest and apoptosis is the main cause of DTX-induced cytotoxicity in PCa cells. To investigate the effects of CXCL12/CXCR4 signaling on the DTX-induced microtubule polymerization and subsequent G2/M phase cell cycle arrest, PCa cells (C4-2 and PC3) were treated for 1h with CXCL12 (100 ng/ml) prior to the treatment of DTX (20 nm). Thereafter, distribution of cells in different phase of cell cycle was analyzed by flow cytometry. Our cell cycle data show an arrest of PCa cells in G2/M phase of cell cycle

upon DTX treatment, which was released in the cells treated with CXCL12. Furthermore, effects of CXCL12 were reversed in PCa cells pre-treated with AMD3100 (**Figure 4**).

To test the hypothesis that activation of CXCL12-CXCR4 signaling axis relieves DTX-induced G2/M phase arrest by counteracting the effects of DTX on the microtubules polymerization, we examined the microtubule polymerization by

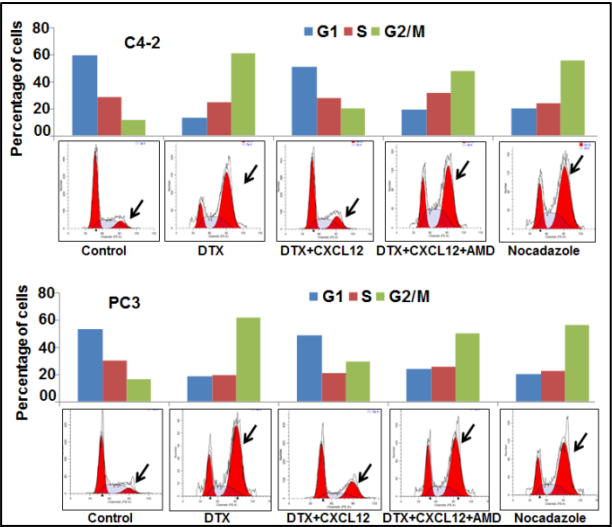


Figure 4: CXCL12/CXCR4 releases docetaxel (DTX)-induced G2/M cell cycle arrest. Synchronized cultures of prostate C4-2 and PC-3 cells were treated with DTX alone or in combination with pre-treatment of AMD3100 and/or CXCL12. After 24h of treatment cells were fixed and, distribution of cells in different phases of cell cycle was analyzed by propidium iodide (PI) staining followed by flow cytometry. Data show a G2/M phase-arrest in DTX-treated cells. Whereas, pre-treatment of CXCL12 abrogated DTX-induced G2/M arrest, which was reversed in the cells pre-treated with AMD3100. Nocadazole was used as positive control.

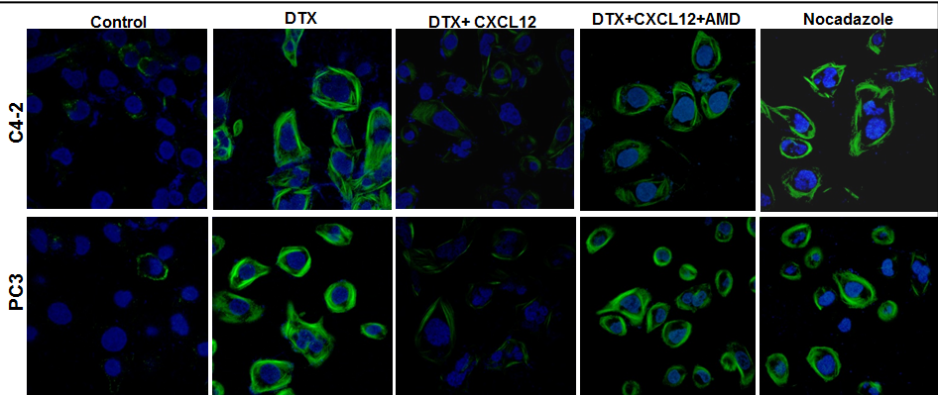
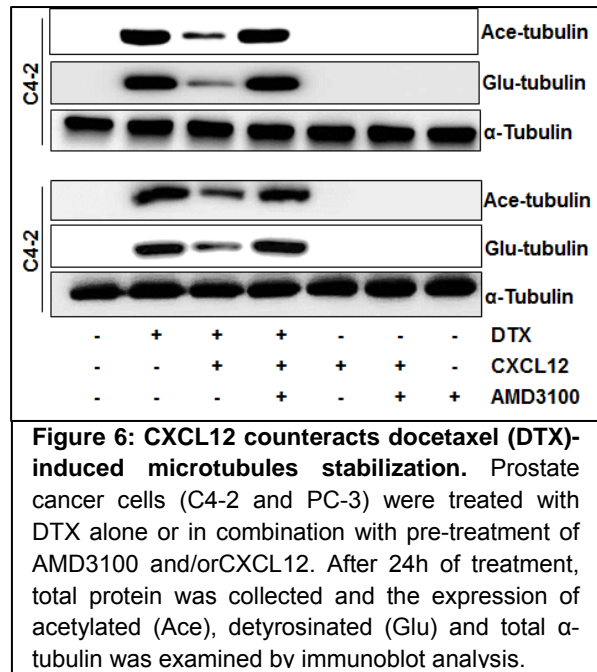


Figure 5: CXCL12 treatment counteracts docetaxel (DTX)-induced microtubules polymerization. Prostate cancer cells (C4-2 and PC-3) were grown on glass bottom plate and treated with DTX alone or in combination with pre-treatment of AMD3100 and/or CXCL12. After treatment cells were fixed, stained with Glu-tubulin, a specific marker of microtubules polymerization, and examined under confocal microscope. Data show increased polymerization of microtubules, which was depicted by the increased expression of Glu-tubulin, in the cells treated with DTX alone. Whereas, pre-treatment of CXCL12 abrogated DTX-induced microtubules polymerization, which was diminished in the cells pre-treated with AMD3100. Cells treated with nocadazole were served as positive control.

we examined the microtubule polymerization by

immunofluorescence analysis using detyrosinated (glu-) tubulin antibody, a specific marker of the polymerized tubulin. As expected, our data revealed enhanced polymerization of microtubules in DTX-treated prostate cancer cells (**Figure 5**). This effect was suppressed when the PCa cells were co-treated with CXCL12- an effect that was abolished in cells pre-treated with AMD3100 (**Figure 5**). In accordance with this, immunoblot analyses demonstrated increased levels of detyrosinated (glu) and acetylated (ace) tubulins, specific markers of the polymerized tubulin, in prostate cancer cells treated with DTX (**Figure 6**). Glu- and Ace- tubulins levels were suppressed in cells co-treated with CXCL12, and regained in cells that were pre-treated with AMD3100 (**Figure 6**).



KEY RESEARCH ACCOMPLISHMENTS:

- We have provided experimental evidence (*in vitro*) for the chemoprotective role of CXCL12/CXCR4 signaling in docetaxel-induced cytotoxicity in prostate cancer cells.
- We have explored the mechanistic insight of the CXCL12/CXCR4-induced chemoresistance in prostate cancer cells. Our data indicate that CXCL12/CXCR4 rescue prostate cancer cells from DTX-induced cytotoxicity by counteracting DTX-induced microtubules polymerization and subsequent G2/M cell cycle arrest in prostate cancer cells.

ONGOING WORK:

- Examining the rescue effect of CXCL12 on docetaxel-induced cytotoxicity by measuring the apoptosis.
- Further confirming the role of CXCL12/CXCR4 signaling in docetaxel-resistance of prostate cancer cells by using CXCR4-targeted siRNA.
- Preparation of the manuscript for publication.

REPORTABLE OUTCOME

We have submitted an abstract entitled “A novel CXCR4-mediated mechanism of docetaxel resistance in prostate cancer cells” for the upcoming AACR (American Association for Cancer Research, April 2013) meeting to present our preliminary findings.